Received: 2 July 2018 | Revised: 8 November 2018 | Accepted: 27 November 2018

DOI: 10.1002/humu.23692

RESEARCH ARTICLE

WILEY HGVS HUMAN GENOME VARIATION SOCIETY

The effect of premature termination codon mutations on *CFTR* mRNA abundance in human nasal epithelium and intestinal organoids: a basis for read-through therapies in cystic fibrosis

Luka A. Clarke[1] | Nikhil T. Awatade[1] | Veronica M. Felício[1] | Iris A. Silva[1] | Maite Calucho[2] | Luisa Pereira[3] | Pilar Azevedo[3] | José Cavaco[4] | Celeste Barreto[3] | Carmen Bertuzzo[5] | Silvia Gartner[2] | Jeffrey Beekman[6] | Margarida D. Amaral[1]

[1]Faculty of Sciences, BioISI - Biosystems & Integrative Sciences Institute, University of Lisboa, Campo Grande, Portugal

[2]Pediatric Pulmonology & CF Unit. Hospital Universitari, Vall d'Hebron, Spain

[3]Department of Pediatrics, Hospital de Santa Maria, Lisboa, Portugal

[4]Centro de Referência de Fibrose Quística, Hospital de Dona Estefânia, Centro Hospitalar de Lisboa Central, Lisboa, Portugal

[5]Human Genetics Department, Faculty of Medical Sciences, University of Campinas, Campinas, Brazil

[6]Department of Pediatric Pulmonology, UMCU, Utrecht, Netherlands

Correspondence
Luka A. Clarke, University of Lisboa, Faculty of Sciences, BioISI - Biosystems & Integrative Sciences Institute, Campo Grande, C8 bldg, 1749-016 Lisboa, Portugal.
Email: laclarke@fc.ul.pt

Funding information
Fundação para a Ciência e a Tecnologia, Grant/Award Numbers: SFRH/BD/87478/2012, SFRH/PD/BD/52487/2014, UID/MULTI/04046/2013; Cystic Fibrosis Foundation Therapeutics, Grant/Award Numbers: AMARAL15XX0, AMARAL15XX1

Communicated by Garry R. Cutting

Abstract

A major challenge in cystic fibrosis (CF) research is applying mutation-specific therapy to individual patients with diverse and rare CF transmembrane conductance regulator (*CFTR*) genotypes. Read-through agents are currently the most promising approach for Class I mutations that introduce premature termination codons (PTCs) into *CFTR* mRNA. However, variations in degradation of PTC containing transcripts by nonsense mediated decay (NMD) might lower read-through efficacy. Allele specific quantitative real time (qRT)-PCR was used to measure variations in *CFTR* mRNA abundance for several PTC mutations in respiratory cells and intestinal organoids. The majority of PTC mutations were associated with reduced levels of relative mRNA transcript abundance (~33% and 26% of total *CFTR* mRNA in respiratory cells and intestinal organoids, respectively, compared to >50% for non-PTC causing mutations). These levels were generally not affected by PTC mutation type or position, but there could be twofold variations between individuals bearing the same genotype. Most PTC mutations in *CFTR* are subject to similar levels of NMD, which reduce but do not abolish PTC bearing mRNAs. Measurement of individual NMD levels in intestinal organoids and HNE cells might, therefore, be useful in predicting efficacy of PTC read-through in the context of personalized CFTR modulator therapy.

KEYWORDS
cystic fibrosis, NMD, personalized therapies, PTC, read-through

1 | INTRODUCTION

A major challenge in current cystic fibrosis (CF) research is developing CF transmembrane conductance regulator (*CFTR*) mutation class-specific therapies (Veit et al., 2016). Currently, six classes of mutations that may be susceptible to CFTR drug-based rescue are recognized plus a seventh class for which CFTR pharmacological rescue is unlikely (De Boeck & Amaral, 2016). A significant proportion of the ~2000 potentially disease causing *CFTR* variants identified to date are Class I mutations, i.e., those introducing an in-frame premature termination codon (PTC) into the *CFTR* messenger RNA (CFTR1 mutation database: www.genet.sickkids.on.ca). However, besides these in-frame nonsense mutations (8% of the total in CF) PTCs can also be introduced by frameshift or splicing mutations (Oren, Pranke, Kerem, & Sermet-Gaudelus, 2017; Sanz et al., 2010), representing approximately 2% and 12% of CFTR mutations, respectively. Translation of an mRNA transcript bearing a PTC will produce a truncated and most likely non-functional protein, but this is largely avoided by an mRNA surveillance mechanism termed nonsense mediated decay (NMD, Frischmeyer & Dietz, 1999). NMD is an important posttranscriptional quality control

mechanism that reduces the abundance of PTC-transcripts (Maquat, 1995), thereby avoiding synthesis of potentially toxic truncated proteins and associated cellular stress in the form of the unfolded protein response (UPF; Walter & Ron, 2011). The absence of functional CFTR protein resulting from PTCs translates into severe CF phenotypes that are usually associated with these mutations (Keeling, Wang, Conard, & Bedwell, 2012) comparable to those of the most common *CFTR* mutation, F508del (c.1521_1523delCTT; Shoshani et al., 1992).

One potential treatment for PTC mutations is the use of read-through agents such as aminoglycoside antibiotics like gentamycin or tobramycin (Hermann, 2007), which can promote insertion of near-cognate amino acids in place of the PTC (Sermet-Gaudelus & Namy, 2016), thereby allowing translation of a full-length and potentially functional protein. However, the reduced abundance of PTC-bearing mRNAs by NMD might reduce the effectiveness of PTC read-through therapies by removing their substrate (Linde, Boelz, Nissim-Rafinia, et al., 2007). Indeed, variations in NMD efficiency have been reported among different cell types and genes (Linde, Boelz, Neu-Yilik, Kulozik, & Kerem, 2007b). The effect of NMD might also vary according to the position and/or sequence context of a specific mutation: for example, PTCs located less than 50–55 nt upstream of the last exon–exon junction, or in close proximity to the normal initiation or termination codons may fail to trigger NMD (Inácio et al., 2004; Nagy & Maquat, 1998).

In this study, we determined the extent to which a variety of *CFTR* PTC-causing mutations affect mRNA abundance via NMD in an allele specific manner in native human nasal epithelial (HNE) cells, so as to unravel whether mRNA levels vary according to mutation type, position or even among different individuals with the same mutation. We also assessed the suitability of intestinal organoids as models for assessing this phenomenon, by comparison with respiratory cells/tissues. Our data provide a basis for the development of read-through therapies targeting Class I mutations in *CFTR*.

2 | MATERIALS AND METHODS

2.1 | Nomenclature

In the present work, the legacy mutation names are given (CFTR1: www.genet.sickkids.on.ca) followed upon first use by the HGVS cDNA name in brackets, based on the *CFTR* reference sequence NM_000492.3. In tables, both legacy names and HGVS variant nomenclature are given for clarity.

2.2 | Participants and nasal respiratory epithelial cell collection

The study was conducted at the Faculty of Sciences of the University of Lisboa with samples collected at the Adult and Pediatric CF Clinics of the Hospital de Santa Maria in Lisboa, and was approved by the Santa Maria Hospital Ethical Review Board. Informed consent for both nasal brushing and rectal biopsy was obtained from each participant, or parent/tutor where the participant was a minor. To be eligible for the study, individuals with CF with PTC causing mutations in *CFTR* were selected, either homozygous or in heterozygosity with F508del or other mutations (PTC/PTC, PTC/F508del, or PTC/other, respectively). Samples from individuals with other genotypes (with non PTC mutations: F508del/other, F508del/F508del, WT/WT, or other/other) were also analyzed, as control samples. HNE cell samples were also sent to Lisboa from two other participating centres (Pediatric Pulmonology & CF Unit, Hospital Universitari, Vall d'Hebron, Barcelona, Spain; Human Genetics Department, Faculty of Medical Sciences, University of Campinas, Brazil) as Trizol lysates (Thermofisher Scientific) on dry ice, having been collected and processed in the same way, i.e., by bilateral brushing (Paro Isola Long 3 mm, Paro, Esro AG, Switzerland) of the nasal mucosa, as previously described (Beck et al., 1999; Harris et al., 2004). In some cases, an aliquot was set aside for conditional reprogramming culture (CRC-HNE), and the remainder was lysed in Trizol reagent for extraction of total RNA.

2.3 | Conditionally reprogrammed cell culture of HNE

Conditional reprogrammed cell (CRC) culture of HNEs was performed as previously reported (Reynolds et al., 2016). Briefly, following brushing the nasal cell samples were washed three times in growth medium and pelleted by gentle centrifugation before addition to a feeder layer of irradiated NIH-3T3 fibroblast cells (ATCC Catalog #CRL-1658) that had been grown to near confluence and irradiated by exposure to a Cobalt 60 gamma ray source (1.60 kGy/h) with a total dose of 31 Gy (3100 rads). The co-culture was maintained in F12 medium containing 10uM Y-27632 Rho Kinase inhibitor. After 1 week of culturing, small islands of epithelial cells appeared among the feeder layer cells. When these epithelial cell islands had grown to cover 90% of the culture dish surface area, differential trypsinization was performed for isolation of the CRC-HNEs. Air-liquid interface (ALI) cultures were then established on 0.4 μM Costar Transwell permeable support filters (Corning) pre-coated with human placental type IV collagen. The cells were seeded at a density of 3×10^5 cells/cm^2 and cultured in F12 medium without Rho Kinase inhibitor or feeder layer. After 5–7 days, when cells had reached confluence on filters, the medium was removed from the apical surface, and the cells were then grown at the ALI for 21 days until polarized. They were then lysed with Trizol and RNA was extracted as described below.

2.4 | Intestinal organoid cultures

Crypt isolation and human organoid culturing were carried out as described previously (Dekkers et al., 2013; Sato & Clevers, 2013; Sato et al., 2009; Sato et al., 2011). Briefly, three to four superficial rectal mucosa specimens (3-4 mm in diameter) were recovered with colon forceps and immediately placed into culture medium. Then, the biopsies were washed with PBS and treated with 10 mM EDTA for 90–120 min at 4ºC. Crypts were isolated by centrifugation and isolated crypts cultured in 50% Matrigel® (growth factor free, phenol-free, BD biosciences). Isolated crypts were seeded (~10–30 crypts in 3×10 μL Matrigel® droplets per well) in prewarmed 24-well plates. The Matrigel® was polymerized for 10–15 min at 37ºC and surrounded

by complete culture medium consisting of: advanced DMEM/F12 supplemented with penicillin/streptomycin, 10 mM HEPES, Glutamax, N2, B27 (all purchased from Invitrogen), 1μM *N*-acetylcysteine (Sigma), and growth factors: 50 ng/mL mEGF, 50% Wnt-3a-conditioning medium (WCM) and 10% Noggin-conditioned medium, 20% Rspo1-conditioned medium (RCM), 10 μM nicotinamide (Sigma), 500 nm A83-01 (Tocris), and 10μM SB 202190 (Sigma). Antibiotics were added to growth medium (Primocin 1:500, Invivogen; Vancomycin and Gentamycin, Sigma) during the first weeks. The medium was changed every alternate day and organoids were passaged after 7–9 days of culturing. Each organoid RNA sample was extracted from three wells.

2.5 | Real time PCR

To determine relative quantitative abundance of *CFTR* transcripts in an allele specific manner, we used the ΔΔCT qRT-PCR method to compare expression of the F508del allele with the other allele by using primer pairs that reliably distinguish between the 3 bp missing (c.1521_1523delCTT) on the F508del allele, as previously described (Awatade et al., 2015). Briefly, RNA was extracted (Trizol method—Invitrogen—or Macherey Nagel Nucleospin columns), quantified by Nanodrop spectrophotometry and digested with DNase I. Approximately 1 μg of total RNA was subjected to reverse transcription (RT) with random primers to produce cDNA (NZYTech, Lisboa, Portugal). cDNA was diluted to give a final reaction mixture concentration between 0.5 and 1.5 ng/μL. Non-F508del and F508del transcripts were then amplified in separate reactions by qRT-PCR in triplicate using the SsoFast EvaGreen system (Bio-Rad, Hercules, CA), with allele specific primers (see Supporting Information Table S1). Melt curves were checked to confirm amplification of single products, and negative controls were confirmed to be free of nonspecific amplification at 40 cycles. Products were quantified using the ΔΔCT method, normalized using as a reference gene either *CAP-1* (adenylate cyclase associated protein 1) or *ACTB* (β actin), and fold change (FC) between F508del and non-F508del products was calculated using the formula $FC = 2^{(-\Delta\Delta CT)}$. Abundance of *CFTR* transcripts derived from each allele was then expressed as a percentage of total *CFTR*. For calculation of "percent transcript degradation" of PTC transcripts, we used the formula: % degradation = ((Y−X)/Y) × 100, where X and Y are percent *CFTR* transcript derived from PTC and non-PTC alleles, respectively, assuming 50% transcript from each allele in the absence of PTC related NMD.

For relative quantification of whole *CFTR* mRNA, a different forward primer, avoiding the F508del mutation, was used (5′-ACTGGAGCAGGCAAGACTTC-3′).

2.6 | PTC mutation specific primers

As many of the PTC samples obtained were not from individuals heterozygous for the F508del mutation, we designed primer sets to distinguish between alleles based on some of the more common PTC mutations encountered, namely E60X (c.178G>T), G542X (c.1624G>T), R553X (c.1657C>T), Y1092X (c.3276C>A), R1162X (c.3484C>T), and W1282X (c.3846G>A). These primer sets were constrained by the position of the nucleotide substitution, and in most cases the single 3′ terminal mismatch was not enough to reliably distinguish between alleles. In order to increase specificity of the PTC primers, a strong mismatch between primer and target sequence (G:A or A:G for all primer sets with the exception of the Y1092X primers, which used a C:C mismatch) was introduced at the -2, -3, or -4 position (with respect to the 3′ terminus) of each primer. The aim was to reduce the overall binding affinity of the 3' region of the primers, while increasing the influence of the final base on target sequence specificity (Kwok et al., 1990). This mismatch amplification mutation assay ("MAMA") has previously been shown to be capable of detecting 30 copies of a mutant allele among 3×10^6 copies of a wt allele (Cha, Zarbl, Keohavong, & Thilly, 1992). The primer sets for PTC mutation discrimination were designed using Primer3 software (Untergasser et al., 2012) with mismatch bases incorporated, are shown in Supporting Information Table S1. Primer specificity was confirmed by control amplification of plasmid *CFTR* DNA.

3 | RESULTS

3.1 | Samples and genotypes

The samples used for this study and their respective genotypes are shown in Supporting Information Table S2. In summary, we used samples from a total of 86 individuals, of which 53 were respiratory cells (mainly freshly collected native HNE cells), and the remainder were intestinal organoid samples derived from 33 individuals. CRC-HNE, organoid, rectal biopsy, and bronchial cell samples were also utilized, derived in a few cases from patients who also provided nasal cell samples. Among the 45 *CFTR* genotypes represented (Supporting Information Table S2), the greatest number were F508del/PTC heterozygous combinations, of which there were 18, the most abundant being G542X/F508del and R1162X/F508del. PTC mutations were also represented in 7 PTC/PTC (e.g., G542X/G542X, W1282X/W1282X, and G542X/R553X) and 11 PTC/other combinations (e.g., p.G542X/R334W [c.1000C>T]). We included some non-PTC *CFTR* genotypes as non-NMD causing controls, and used both WT/WT and F508del/F508del homozygotes as primer controls. We also included a number of CRC-HNE samples, primary HBE cells, and rectal biopsies (i.e., non-cultured) when available.

3.2 | Relative mRNA abundance for PTC mutations in trans with F508del

Data on relative abundance of *CFTR* mRNA as measured by allele specific qRT-PCR using primers discriminating between presence or absence of the F508del mutation in heterozygous samples, are compiled in Table 1 (for respiratory cells, principally native HNEs, but also primary HBE cells and CRC-HNEs) and Table 2 (for intestinal organoids). All these data are also summarized in Figure 1.

As expected, most PTC causing *CFTR* mutations were associated with reduced mRNA abundance, as measured by allele specific qRT-PCR. For example, in HNE samples from 10 CF patients with the

TABLE 1 Relative abundance and percent degradation of non-F508del *CFTR* transcripts in respiratory tissues obtained with F508del primers (mean% ± SEM)

Non-F508del Allele (Legacy/traditional nomenclature)	Non-F508del Allele (HGVS nomenclature[a])	Model (if not native HNE)	*N* (patients)	Percentage of total *CFTR* mRNA represented by PTC or "other" allele (±SEM if *N* patient > 2)	Percent degradation of PTC bearing transcript
PTC causing (assumed)					
S4X	c.11C>A		1	47.8%	8.4%
G542X	c.1624G>T		10	28.7% (±2.6)	59.8%
G542X	c.1624G>T	Primary HBE	1	21%	73.4%
1811+1.6kbA>G	c.1679+1634A>G		1	37.6%	39.7%
2183AA>G	c.2051_2052delAAinsG		3	37.4% (±7.4)	40.3%
Q890X	c.2668C>T		2	38%	38.7%
3272-26A>G	c.3140-26A>G		1	34.9%	46.4%
Y1092X	c.3276C>A	Primary HBE	1	26%	64.9%
3886insA	c.3754_3755insA		1	33%	50.7%
3886insA	c.3754_3755insA	CRC-HNE	1	27%	63%
Other (non PTC)					
P205S	c.613C>T		1	61%	N/A
R334W	c.1000C>T		2	53%	N/A
R334W	c.1000C>T	CRC-HNE	3	50% (±1.2)	N/A
R347P	c.1040G>C	Primary HBE	1	52%	N/A
A561E	c.1682C>A		1	57%	N/A
1812-1G>A	c.1680-1G>A		1	63.7%	N/A

[a]Based on NCBI Reference Sequence for CFTR: NM_000492.3.
Abbreviations: HNE, human nasal epithelial cells; CRC, conditionally reprogrammed HNE cells; HBE, human bronchial epithelial cells.

F508del/G542X genotype, a mean 28.7 ± 2.6% (SEM) of total *CFTR* mRNA was found to be derived from the G542X allele, with the remainder corresponding to *F508del-CFTR* mRNA. Assuming that 50% mRNA is initially transcribed from each allele, it was calculated that on average 59.8% of the transcribed G542X-CFTR mRNA was degraded, most likely by NMD. Among these 10 G542X/F508del samples, abundance of PTC-bearing mRNA varied between 13.5% and 38.2%, supporting previous finding that there are inter-individual differences in NMD, which in turn might influence the effectiveness of PTC read-through agents (Linde, Boelz, Nissim-Rafinia, et al., 2007a).

Similar data were obtained in respiratory tissues for other PTC mutations, including other Class I PTC mutants Q890X (c.2668C>T) and Y1092X, frameshift and splicing mutants 1811+1.6kbA>G (c.1679+1634A>G), 2183AA>G (c.2051_2052delAAinsG), 3272-26A>G (c.3140-26A>G), and 3886insA (c.3754_3755insA), which suffered between 39% and 63% transcript degradation. As expected, non-PTC mutants (P205S [c.613C>T], R334W, A561E [c.1682C>A]) were present at approximately 55% of mRNA abundance with little variation. Interestingly, the S4X (c.11C>A) and 1812-1G>A (c.1680-1G>A) mutants did not show evidence for NMD. Data generated using primary HBE cells and CRC-HNEs showed no significant differences from those in native HNEs, and can be considered equivalent.

Table 2 presents the combined results from intestinal organoid samples, which had F508del on the second allele. As seen in respiratory tissues, PTC causing *CFTR* mutations were also associated with reduced mRNA abundance in organoids (from 17.4% for Y1092X to 39% for E60X, with 79% and 36% of PTC-bearing transcripts degraded, respectively). Once again, the splicing mutants 2789+5G>A (c.2657+5G>A), 3849+10kbC>T (c.3717+12191C>T), and 3500–2A>G (c.3368-2A>G) were associated with a similar level of transcript degradation (around 70% in each case), which is to be expected, as these splicing defects introduce PTCs following a shift in reading frame in the aberrantly spliced mRNA. This was, however, not the case for the 711+1G>T (c.579+1G>T) mutation, which does not cause a frameshift. The dele2,3_21 kb (c.54-5940_273+10250del21kb) mutation, which was predicted to cause a reading frame shift with the consequent introduction of a PTC, was not, however, associated with reduced mRNA abundance. As expected, non-PTC mutants (R334W, A455E [c.1364C>A]) were present at around 50% of mRNA abundance, as in respiratory cells. The data presented here confirm that, in general, intestinal organoids appear to be a valid surrogate for the study of allele specific *CFTR* mRNA abundance in respiratory epithelium, as shown by comparison of means in Figure 1.

3.3 | Relative abundance of PTC transcripts estimated using PTC specific primer sets

For the analysis of samples bearing PTC mutations in heterozygosity with non-F508del *CFTR* mutations (i.e., PTC/other or PTC/PTC as opposed to PTC/F508del), we designed primers to discriminate between the six most commonly encountered PTC mutations (E60X, G542X, R553X, R1162X, Y1092X, and W1282X) and the opposing

TABLE 2 Relative abundance and percent degradation of non-F508del *CFTR* transcripts in intestinal organoids obtained with F508del primers (mean% ± SEM)

Non-F508del Allele (Legacy/traditional nomenclature)	Non-F508del Allele (HGVS nomenclature[a])	*N* patient (*n* repl.)	Percentage of total *CFTR* mRNA represented by PTC or "other" allele (±SEM if *N* patient > 2)	Percent degradation of PTC bearing transcript
PTC causing				
E60X	c.178G>T	1 (3)	39%	36.1%
365insT	c.233_234insT	1 (3)	27%	63%
G542X	c.1624G>T	2 (3, 3)	20.4%	74.4%
2183AA>G	c.2051_2052delAAinsG	1 (4)	20%	75%
E730X	c.2188G>T	1 (1)	26.4%	64.1%
W846X	c.2537G>A	1 (1)	37%	41.3%
Y1092X	c.3276C>A	1 (3)	17.4%	78.9%
R1162X	c.3484C>T	4 (3,3,3,3)	27.3% (±3.6)	62.4%
W1282X	c.3846G>A	2 (3,3)	26%	64.9%
Intronic / Splicing				
711+1G>T	c.579+1G>T	2 (3,2)	52.5%	N/A
2789+5G>A	c.2657+5G>A	3 (3,3,1)	21.7% (±1.2)	72.3%
3849+10kbC>T	c.3717+12191C>T	1 (1)	23%	70.1%
3500-2A>G	c.3368-2A>G	1 (3)	23%	70.1%
Other (non PTC)				
dele2,3_21 kb	c.54-5940_273+10250del21kb	1 (3)	54%	N/A
A455E	c.1364C>A	1 (3)	49%	N/A
R334W	c.1000C>T	2 (2, 2)	55%	N/A

[a]Based on NCBI Reference Sequence for CFTR: NM_000492.3.

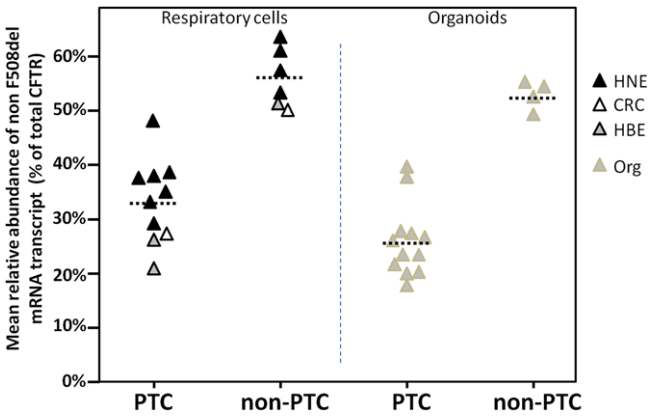

FIGURE 1 Mean relative abundance of non F508del *CFTR* transcripts with or without PTC mutations, in respiratory cells (fresh and cultured) versus organoids. Means of relative CFTR mRNA abundance for non-F508del transcript as measured by qRT-PCR using F508del primer set are shown: data is summarized from Tables 1 and 2. Each triangle represents the mean value for one mutation and one cell type (as shown in key). Group means were 33.06% ($n = 10$) and 55.95% ($n = 6$) for PTC and non-PTC bearing respiratory cells, and 25.68% ($n = 12$) and 52.63% ($n = 4$) for PTC and non-PTC bearing intestinal organoids, respectively

allele (see Methods and Supporting Information Table S1). These primers had lower degrees of allele specificity than the F508del specific primers: the proportion of nonspecific amplification of *wt-CFTR* plasmid DNA by mutation specific primers compared to the respective wt primers after 40 cycles of qRT-PCR was 1:11666 for the F508del primer set, but lower for E60X (1:6841), G542X (1:189), W1282X (1:128), R1162X (1:117), Y1092X (1:85), and R553X (1:17) primers.

Data obtained from the PTC mutation specific primers (Supporting Information Table S3) generally supported the data obtained using the F508del primer set, but with some differences. In some cases (F508del/PTC genotypes), it was possible to apply both primer sets to the same samples. Some such data were very similar (e.g., G542X/F508del HNE data and W1282X/F508del organoid data), thereby validating the PTC-specific primers. Comparison of a set of HNE samples bearing the G542X/F508del genotype for which data were obtained for both F508del and G542X specific primers showed no significant difference ($n = 6$, mean% ± SEM) for F508del primers = 30.2 ± 3.9%, mean for G542X primers = 25.5 ± 3.5%: see Figure 2a).

In other cases, however, the PTC specific primer sets underestimated (e.g., R1162X/F508del and Y1092X/F508del organoid data) or overestimated (e.g., E60X/F508del organoid data) the quantification data provided by the F508del primers (see Figure 2b).

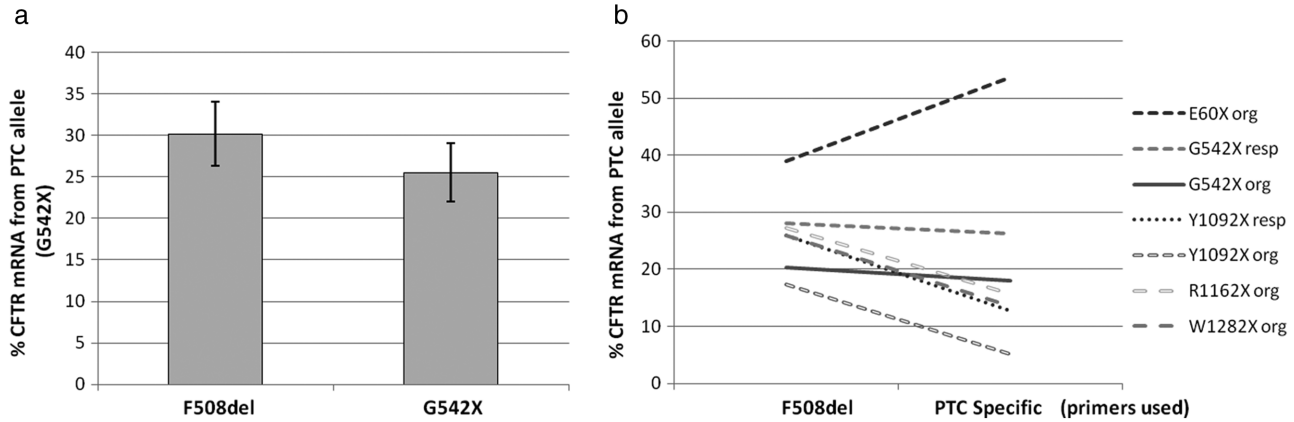

FIGURE 2 Relative abundance of PTC bearing *CFTR* transcripts estimated using F508del and PTC primer sets. (a) Allele-specific *CFTR* mRNA abundance was measured for the same set of HNE samples with the F508del/G542X genotype ($n = 6$). Mean ± SEM are shown (30.2 ± 3.9% for F508del primers, 25.5 ± 3% for G542X primers: not significantly different). (B) Other PTC primer sets vary in their relative efficiencies. F508del/PTC samples were amplified for samples with E60X, G542X, Y1092X, R1162X, and W1282X CFTR, using F508del primers and the respective PTC primer sets. Abundance of mRNAs derived from PTC alleles are similar to that of F508del/G542X samples, but E60X primers overestimate, while R1162X and Y1092X primers underestimate values given by F508del primers due to different relative efficiencies. Data here are compiled from Tables 1 and 2 and Supporting Information Table S3, and do not necessarily represent the same samples for alternative primer sets

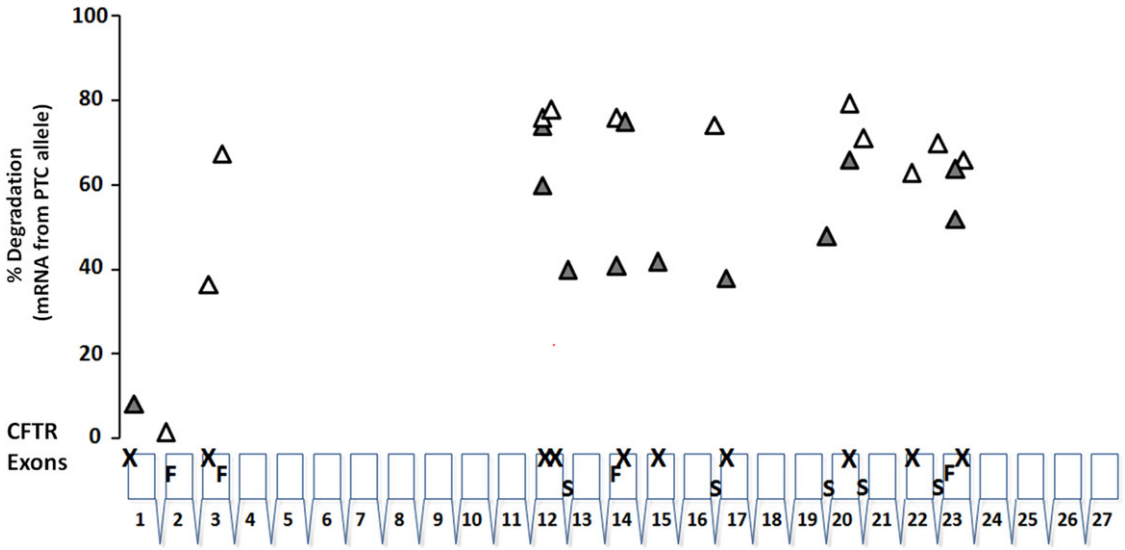

FIGURE 3 Effect of position of PTC mutations along *CFTR* gene on relative abundance of PTC-transcripts. Percentage degradation of mRNA derived from PTC bearing alleles plotted against position of PTC causing mutations (nonsense: X, frameshift: F, or Splicing: S) in *CFTR* gene. Only data points from Tables 1 and 2, obtained using F508del primers either in respiratory cells (dark triangles) or organoids (clear triangles) are shown. The order of mutations shown is as follows: S4X (X, ex. 1), dele2,3_21 kb (F, ex. 2), E60X (X, ex. 3), 365insT (F, ex. 3), G542X (X, ex. 12), R553X (X, ex. 12), 1811+1.6kbG>A (S, intr. 12), 2183AA>G (F, ex. 14), E730X (X, ex. 14), W846X (X, ex. 15), 2789+5G>A (S, intr. 16), Q890X (X, ex. 17), 3272-26A>G (S, intr. 19), Y1092X (X, ex. 20), 3500−2A>G (S, intr. 20), R1162X (X, ex. 22), 3849+10kbC>T (S, intr. 22), 3886insA (F, ex. 23), W1282X (X, ex. 23)

3.4 | Influence of PTC position on mRNA abundance

Measured levels of mRNA abundance were not influenced by whether the PTC resulted from a nonsense, frameshift, or splicing mutation. Next, we assessed whether either the location of each PTC causing mutation along the *CFTR* gene (Figure 3) might be a factor influencing NMD. A distance of less than 50–55 nt to the last exon–exon junction has been reported to enable NMD avoidance (Nagy & Maquat, 1998). However, among the tissues available for testing, none had mutations occurring in the penultimate exon. The only mutations giving any indication that they might escape NMD to some degree were S4X, dele2,3_21 kb, and possibly E60X, each of which could be associated with the use of an alternative initiation codon, such as met150 (Ramalho et al., 2009).

3.5 | Whole *CFTR* mRNA abundance versus *CFTR* genotype

In order to determine whether the observed reduction in abundance of *CFTR* PTC-RNAs caused an overall reduction in *CFTR*

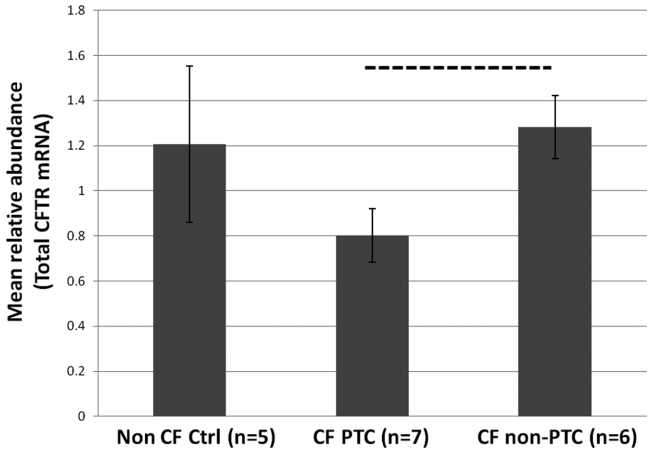

FIGURE 4 Whole *CFTR* abundance measured in HNE cells grouped by mutation type. Relative expression of whole *CFTR* mRNA was measured in native HNE cells from healthy controls, CF patients with one PTC causing *CFTR* allele and CF patients with no PTC causing allele (mean ± SEM). There was a significant ($P < 0.05$) reduction of *CFTR* mRNA for the CF-PTC group compared to the CF-non-PTC group (dotted line)

mRNA abundance, relative expression of total *CFTR* mRNA was measured in native HNE cells from healthy controls (Non-CF Ctrl; $n = 5$), CF patients with one PTC causing allele (CF-PTC; $n = 7$: genotypes G542X/F508del; 2183AA>G/F508del; 3886insA/F508del; Y1092X/P205S; 2x W1282X/H1079P; 3849+10kbC>T/F508del), and CF patients with no PTC causing allele (CF-non PTC; $n = 6$: genotypes A561E/F508del; 2x R334W/F508del; P205S/F508del; R560S[c.1680A>C]/R560S; F508del/F508del). As shown in Figure 4, there was indeed a significant reduction (approximately 38%, $P < 0.05$) of total *CFTR* mRNA for the CF-PTC group compared to the CF-non PTC group, representing 76% degradation of mRNA derived from the PTC allele, in agreement with our observations for individual mutations.

4 | DISCUSSION

Using an allele specific qRT-PCR approach, the present study demonstrates that abundance of mRNA derived from *CFTR* alleles bearing PTC causing mutations is reduced compared to that derived from non-PTC alleles, and generally PTC-mRNA levels are just around 21–38% of the total *CFTR* mRNA in native HNE cells, with similar levels (17–39%) seen in intestinal organoids. The observed range of reduction is similar for transcripts with PTCs introduced by nonsense, frameshift, or splicing mutations, and is independent of mutation position, both within the *CFTR* gene and, for most nonsense mutations, within the exon, regarding the distance to the next downstream exon–exon junction. There is, however, considerable variation in PTC mRNA abundance among individuals with the same PTC mutation, for example, a range of 13.5–38.2% for HNE samples with the G542X/F508del genotype from 10 different individuals.

These results are consistent with previous estimates that NMD reduces the abundance of PTC containing mRNAs to from 5% to 25% of non-PTC transcripts (Kuzmiak & Maquat, 2006). It is also known that NMD varies among patients bearing distinct *CFTR* mutations (Linde, Boelz, Nissim-Rafinia, et al., 2007a), and that the efficiency of NMD may also vary between cell types (Linde, Boelz, Neu-Yilik, et al., 2007b). More recently it has been shown that NMD factors may be suppressed during differentiation, which may be of relevance to NMD in airway epithelium (Lou et al., 2016), and it is becoming clear that NMD is not just an RNA surveillance machinery eliminating PTCs, but also a posttranscriptional gene expression regulation system that selectively degrades mRNAs implicated in various specific cell functions (Han et al., 2018). Our data suggest that in the case of PTC mutations in *CFTR*, there is a balance between NMD and gene transcription, which favors reduced accumulation, but not outright elimination of the PTC bearing mRNA transcripts. A restricted transcriptional shutdown experiment confirmed that *CFTR* mRNA bearing the G542X PTC has a lower half-life compared to F508del in intestinal organoids (Supporting Information Figure S1), as expected. The extent to which this reduced stability varies between PTCs, and how such variations might specifically hamper read-through efficacy has yet to be tested, but the outcome is that for most PTC causing mutations a considerable amount of transcript is still available. These transcripts might thus be translated into truncated protein, of some potential toxicity to the cell. However, in turn they may also serve for PTC read-through based therapeutic approaches, a fact which is of relevance to the future application of *CFTR* Class I mutation correction strategies.

The broad agreement between values obtained in HNEs and intestinal organoids contributes to validating the use of the latter model as a surrogate for the respiratory epithelium in studies of *CFTR* gene expression and NMD, and by extension for testing therapeutic approaches as already demonstrated for Class I mutations (Zomer-van Ommen et al., 2016) and other mutation classes (Dekkers et al., 2016). Nevertheless, it can be noticed that slightly higher levels of PTC degradation were measured in organoids than in HNE (see Figures 1 and 3), and it would be interesting to know if this correlates with the specific relative expression of NMD factors in these cell types.

Some mutations produced apparently discrepant data: S4X and possibly E60X appear to escape NMD-related degradation, which could be the result of alternative initiation codon usage as previously suggested for other mutations occurring in the region around the initiation codon (Ramalho et al., 2009). However, for S4X, it is more likely to be by virtue of its position in the fourth codon of *CFTR*, in close proximity to the start codon, which itself is a major determinant of NMD avoidance (Inácio et al., 2004). On the other hand, the large deletion mutant dele2,3_21 kb, predicted to cause a frameshift and a PTC early in exon 4 was also found here to evade enhanced degradation. Interestingly, this was also observed in an original study of this mutation in HNE samples (Dörk et al., 2000), which suggested re-initiation of translation at an internal AUG codon at nt position 150 in exon 4, similarly to the above situation, as a possible explanation for the lack of degradation of this transcript. Other data served to confirm the relationship between PTCs and NMD: the 1812-1G>A and 711+1G>T splicing mutations are associated with skipping of exons 13 and 5, respectively (Felício, Ramalho, Igreja, & Amaral, 2017; Fonknechten, Chomel, Kitzis, Kahn, &

Kaplan, 1992). As these two exons possess 'whole numbers' of codons (i.e., nucleotide numbers that are multiples of 3), neither the introduction of PTCs nor the consequent triggering of NMD are expected.

The values obtained for relative abundance of non-PTC mutations (including P205S, R334W, and A561E) in heterozygosity with F508del in both HNEs and organoids are generally higher than 50% of total *CFTR* mRNA (see Figure 1), suggesting that *F508del-CFTR* mRNA is itself subject to a reduction in abundance. The average ratio of F508del/non-PTC transcripts is 79% (HNE) and 90% (organoids), similar to a figure of 87% measured in HNE that we have previously reported (Ramalho et al., 2002). It should therefore be borne in mind that abundance of mRNA from PTC alleles as presented here is possibly overestimated when measured against the F508del allele. This may help explain why some values obtained using mutation specific primers seem low compared to values for the same mutations using F508del primers (see Supporting Information Table S3). Further optimization of the PTC mutation specific primers is desirable: each primer set has a specific level of substrate efficiency, shown by different ratios of *wt-CFTR* plasmid amplification by WT versus mutant primers. Improvements in specificity might be achieved by introduction of additional or alternative mismatches into the central primer regions. However, sequencing based methods such as RNAseq might also provide an alternative quantitative approach applicable for any combination of CFTR genotypes.

We have shown that mRNAs transcribed from PTC bearing *CFTR* alleles occur with a consistently reduced abundance compared to non-PTC alleles, presumably being subject to NMD. This is equally the case in native HNE cells and cultured intestinal organoids derived from rectal biopsies. Since the precise values of relative PTC-mRNA abundance were subject to twofold variations between individuals with the same *CFTR* genotype, we propose that individual NMD levels would be useful in predicting the efficacy of mutation-specific CFTR modulator therapies, namely those using read-through approaches. Furthermore, intestinal organoids and HNE cells would be equally suitable for such measurements. Recent work confirmed that G418 read-through induced limited CFTR function for E60X/4015delATTT organoids lacking residual CFTR function (Zomer-van Ommen et al., 2016). This was enhanced by CFTR modulators VX-770/809 confirming that even unstable PTC mRNA can be sufficient for successful read-through by pharmacological agents, especially if further boosted by correctors and potentiators. However, future work on PTC mutation specific transcript stability is important to identify the variants most likely to benefit from these approaches.

ACKNOWLEDGMENTS

Work supported by UID/MULTI/04046/2013 center grant from FCT/MCTES, Portugal (to BioISI) and CFF-Cystic Fibrosis Foundation USA research grants to M.D.A. (AMARAL15XX0, AMARAL15XX1-"RNA Life"). V.M.F. and N.T.A. were recipients of PhD fellowships SFRH/BD/87478/2012 and SFRH/PD/BD/52487/2014 (the latter from BioSys PhD programme) from F.C.T., Portugal. The authors thank Maria Manuela Coelho (DBA) for use of Q-PCR machine. The authors also thank Luciana Rezende for cell/RNA/cDNA processing, and Sandra Cabo Verde and Pedro Santos (ITN, Lisboa), for feeder cell irradiation.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ORCID

Luka A. Clarke https://orcid.org/0000-0003-3254-9121

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SUPPORTING INFORMATION

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How to cite this article: Clarke LA, Awatade NT, Felício VM, et al. The effect of premature termination codon mutations on *CFTR* mRNA abundance in human nasal epithelium and intestinal organoids: a basis for read-through therapies in cystic fibrosis. *Human Mutation*. 2019;40:326–334. https://doi.org/10.1002/humu.23692